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(54) Title: ASSAY METHOD

(57) Abstract: An assay is provided for the identificaiton and compariosn of compounds haivng activity as modulators of the ex-
pression and release of TNF α , IL-1 or IL-6 in humans and animals in whichmodulation of the catalytic activity of PRAK
(p38-regulated/activated prtein kinase) is employed for identification and comparision of test compounds.

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ASSAY METHOD

Government Rights

This invention was made with government support under Grant GM51417, AI41637 and AHA95007690 awarded by the National Institutes of Health. The U.S. Government has certain rights in the invention.

Field of the Invention

This invention relates to biologically active compounds, in particular to compounds which have activity as modulators of the expression and release of TNF α , IL-1 or IL-6 in humans and animals, to processes for the identification of such compounds and also to the therapeutic use of such compounds for the treatment of TNF α , IL-1 or IL-6 mediated diseases such as rheumatoid arthritis and diseases of bone metabolism, e.g. osteoporosis.

Background of the Invention

PRAK (p38-regulated/activated protein kinase) is a recently discovered protein kinase which is regulated by p38 MAP kinase, and was first described by New et al. (New L., Jiang Y., Zhao M., Liu K., Zhu W., Flood L. J., Kato Y., Parry G. C. N. and Han J.; EMBO Journal, Vol. 17, No. 12, pp. 3372-3384, 1998). New et al. report that activated PRAK is able to phosphorylate small heat shock protein 27 (HSP27) at the physiologically relevant sites, but indicate that the question of whether PRAK is a physiologically relevant kinase for small heat shock proteins awaits further investigation.

We have now found that small molecular weight inhibitors of the catalytic activity of PRAK in vitro inhibit the production of the inflammatory mediators TNF α , IL-1 or IL-6.

For ease of reference, compounds which have activity as modulators of the expression and release of TNF α , IL-1 or IL-6 in humans and animals are hereinafter referred to as TNF Modulators

Description of the Invention

Accordingly in a first aspect the invention provides use of PRAK for the identification of a TNF Modulator.

In particular, the invention may be used for the identification of TNF Modulators which are inhibitors of PRAK.

Thus in a preferred embodiment the first aspect of the invention provides a method for the identification of a TNF Modulator which comprises contacting a test compound with a system comprising a PRAK protein and monitoring the system for inhibition of PRAK catalytic activity.

For the purposes of the present description "PRAK protein" means a full length human or animal PRAK, e.g. the 471 amino acid human PRAK protein described by New et al. (ibid), or any fragment or analogue thereof having PRAK catalytic activity.

The method of the invention (hereinafter the PRAK Inhibition assay) may be used for screening of individual compounds and for screening of compound collections for TNF Modulators; for example for screening of compound collections or libraries comprises from a few compounds up to tens of thousands of compounds or more, including combinatorial compound libraries. The PRAK inhibition assay may be used as a first line screening assay to identify lead compounds. Alternatively a similar assay may be used to compare and quantify the PRAK inhibition activity of compounds, e.g. to compare compounds produced from medicinal chemistry lead optimization/derivatisation programmes.

Thus in a further aspect the invention provides a method for the comparison of TNF Modulators, comprising separately contacting the TNF Modulators with a system comprising a PRAK protein and comparing each system for inhibition of PRAK catalytic activity (hereinafter referred to as the PRAK Comparison assay).

The compounds for screening, e.g. primary screening, may be synthetic chemical compounds or naturally occurring compounds or compounds extracted from nature or from culture systems, or mixtures of any of these.

Typically the PRAK protein is activated prior to use in the PRAK Inhibition or PRAK Comparison assay. For instance, the PRAK protein is phosphorylated by incubation with an appropriate phosphorylating enzyme, e.g. phospho-p38; for example as hereinafter described in the Example.

The activated PRAK protein is conveniently contacted with test compound in solution. For example, the activated PRAK protein may be prepared in an appropriate buffer, e.g. kinase buffer, and the test compounds dissolved in an appropriate solvent, e.g. DMSO, and the two solutions mixed together with other reagents as required.

Any suitable methodology may be used to monitor for inhibition of PRAK catalytic activity, conveniently by monitoring the absence or decrease in PRAK catalytic activity in the presence of test compound as compared with the absence of test compound. Thus, the catalytic activity of PRAK protein on one or more substrates may be used to indicate PRAK catalytic activity and a complete or partial inhibition of this activity may be used to indicate PRAK inhibition. Preferably the activity of PRAK protein as a kinase for phosphorylation of heat shock protein, e.g. HSP27, may be used to monitor the outcome of the PRAK Inhibition or PRAK Comparison assay.

Thus for example, activated PRAK protein, heat shock protein, e.g. HSP27, and phosphate donor, e.g. ATP, are incubated together in the presence of test compound and the extent of phosphorylation of heat shock protein which results is compared with that which results in the absence of test compound. A complete or partial inhibition of phosphorylation of heat shock protein indicates that the test compound is a TNF Modulator as defined above. Conveniently the extent of phosphorylation of heat shock protein may be determined using appropriate labeling, e.g. labeling of the phosphate donor substrate, e.g. conveniently by use of $\gamma^{33}\text{P}$ -ATP. The amount of labeled material present in the phosphorylated heat shock protein when the assay is conducted in the presence of test compound, as compared with when the assay is conducted in the absence of test compound, is indicative of the inhibitory activity of the test compound.

In a further aspect the invention includes TNF Modulators when identified by the PRAK Inhibition assay or when selected by a method comprising the the PRAK Comparison assay, hereinafter referred to as TNF Modulators of the Invention.

In particular TNF Modulators of the Invention possess PRAK inhibiting activity and thus are indicated as inhibitors of production of inflammatory cytokines, such as $\text{TNF}\alpha$, IL-1 and IL-6, and also to potentially block the effects of these cytokines on their target cells. These and other pharmacological activities of the Agents of the Invention as may be demonstrated in standard test methods for example as described below in the Example.

TNF Modulators of the Invention typically have IC_{50} s for PRAK inhibition of about 1 μM or less, e.g. from about 10 μM to about 100nM or less, e.g. of about 10nM or less.

As indicated in the Example TNF Modulators of the Invention are potent inhibitors of TNF α release. Accordingly, the TNF Modulators of the Invention have pharmaceutical utility as follows:

TNF Modulators of the Invention are useful for the prophylaxis and treatment of diseases or pathological conditions mediated by cytokines such as TNF α , IL-1 or IL-6 e.g., inflammatory conditions, autoimmune diseases, severe infections, and organ or tissue transplant rejection, e.g. for the treatment of recipients of heart, lung, combined heartlung, liver, kidney, pancreatic, skin or corneal transplants and for the prevention of graft-versus-host disease, such as following bone marrow transplants.

TNF Modulators of the Invention are particularly useful for the treatment, prevention, or amelioration of autoimmune disease and of inflammatory conditions, in particular inflammatory conditions with an aetiology including an autoimmune component such as arthritis (for example rheumatoid arthritis, arthritis chronica progrediente and arthritis deformans) and rheumatic diseases. Specific autoimmune diseases for which Agents of the Invention may be employed include autoimmune haematological disorders (including e.g. hemolytic anaemia, aplastic anaemia, pure red cell anaemia and idiopathic thrombocytopenia), systemic lupus erythematosus, polychondritis, scleroderma, Wegener granulomatosis, dermatomyositis, chronic active hepatitis, myasthenia gravis, psoriasis, StevenJohnson syndrome, idiopathic sprue, autoimmune inflammatory bowel disease (including e.g. ulcerative colitis and Crohn's disease), endocrine ophthalmopathy, Graves disease, sarcoidosis, multiple sclerosis, primary biliary cirrhosis, juvenile diabetes (diabetes mellitus type I), uveitis (anterior and posterior), keratoconjunctivitis sicca and vernal keratoconjunctivitis, interstitial lung fibrosis, psoriatic arthritis and glomerulonephritis (with and without nephrotic syndrome, e.g. including idiopathic nephrotic syndrome or minimal change nephropathy).

TNF Modulators of the Invention are also useful for the treatment, prevention, or amelioration of asthma, bronchitis, pneumoconiosis, pulmonary emphysema, and other obstructive or inflammatory diseases of the airways.

TNF Modulators of the Invention are useful for treating undesirable acute and hyperacute inflammatory reactions which are mediated by TNF, especially by TNF α , e.g., acute infections, for example septic shock (e.g., endotoxic shock and adult respiratory distress syndrome), meningitis, pneumonia; and severe burns; and for the treatment of

cachexia or wasting syndrome associated with morbid TNF release, consequent to infection, cancer, or organ dysfunction, especially AIDS -related cachexia, e.g., associated with or consequential to HIV infection.

TNF Modulators of the Invention are particularly useful for treating diseases of bone metabolism including osteoarthritis, osteoporosis and other inflammatory arthritides. For the above indications the appropriate dosage will, of course, vary depending, for example, on the particular TNF Modulator of the Invention employed, its activity, the subject to be treated, the mode of administration and the nature and severity of the condition being treated.

The TNF Modulators of the Invention may be administered by any conventional route, e.g. orally, for example in the form of solutions for drinking, tablets or capsules or parenterally, for example in the form of injectable solutions or suspensions. Normally for systemic administration oral dosage forms are preferred, although for some indications the TNF Modulators of the Invention may also be administered topically or dermally, e.g. in the form of a dermal cream or gel or like preparation or, for the purposes of application to the eye, in the form of an ocular cream, gel or eyedrop preparation; or may be administered by inhalation, e.g., for treating asthma.

The invention includes the use of the TNF Modulators of the Invention for the prophylaxis and treatment of diseases or pathological conditions mediated by cytokines such as TNF α , IL-1 or IL-6.

The invention includes:

- i) A method of inhibiting production of soluble TNF, especially TNF α , or of reducing inflammation in a subject (i.e., a mammal, especially a human) in need of such treatment which method comprises administering to said subject an effective amount of a TNF Modulator of the Invention, or a method of treating any of the above mentioned conditions, particularly a method of treating an inflammatory or autoimmune disease or condition, e.g. rheumatoid arthritis, or alleviating one or more symptoms of any of the above mentioned conditions.
- ii) A TNF Modulator of the Invention for use as a pharmaceutical, e.g. for use as an immunosuppressant or antiinflammatory agent or for use in the prevention, amelioration or treatment of any disease or condition as described above, e.g., an autoimmune or inflammatory disease or condition.

iii) A pharmaceutical composition comprising a TNF Modulator of the Invention in association with a pharmaceutically acceptable diluent or carrier, e.g., for use as an immunosuppressant or anti-inflammatory agent or for use in the prevention, amelioration or treatment of any disease or condition as described above, e.g., an autoimmune or inflammatory disease or condition.

iv) Use of a TNF Modulator of the Invention in the manufacture of a medicament for use as an immunosuppressant or anti-inflammatory agent or for use in the prevention, amelioration or treatment of any disease or condition as described above, e.g., an autoimmune of inflammatory disease or condition.

In a yet further aspect the invention includes TNF Modulators which are specific inhibitors of PRAK (hereinafter referred to as Specific TNF Modulators) and to the use of such Specific TNF Modulators for the prophylaxis and treatment of diseases or pathological conditions mediated by cytokines such as TNF α , IL-1 or IL-6; for instance, in methods, uses and compositions similar to those defined above in paragraphs i) to iv) for the TNF Modulators of the Invention.

For the purposes of the present description a Specific TNF Modulator is a compound which specifically inhibits PRAK as compared with other kinases of the p38 MAP kinase cascade. Conveniently Specific TNF Modulators inhibit PRAK with an IC₅₀ which is at least about 1 μ M, e.g. from about 10 μ M to about 100nM or less, e.g. of about 10nM or less, and have IC₅₀s for inhibition of other kinases of the p38 MAP kinase cascade, e.g. for inhibition of p38 MAP kinase, which are at least 10 μ M, preferably at least 100 μ M or more.

The invention is further described by way of illustration only in the following Example which relates to assay for *in vitro* PRAK kinase activity, and TNF α , IL-1 β , and IL-6 activity and our finding that small molecular weight inhibitors of the catalytic activity of PRAK inhibit the product of TNF α , IL-1 β , and IL-6 *in vitro*.

EXAMPLE

Inhibitors of PRAK, a downstream kinase of p38a, have anti-inflammatory properties.

Prak was recently identified as a substrate of p38a (New et al. *ibid*). Prak is a kinase homologous to MapkapK2, MapkapK3, Mnk1 and Mnk2, and is able to efficiently phosphorylate the small heat shock protein HSP27 in vitro (New et al. *ibid*). Further in vivo substrates have not been identified yet, and the biological function of Prak is unknown to date.

Below we provide evidence that Prak is involved in the expression of TNF α , IL-1 or IL-6 in human peripheral blood mononuclear cells or dermal fibroblasts, based on the fact that small molecular weight inhibitors of the catalytic activity of Prak inhibit the production of the inflammatory mediators TNF α , IL-1, or IL-6 in vitro. Thus, Prak is apparently involved in the inflammatory response in vivo, and inhibition of this kinase provides a new anti-inflammatory principle.

Materials and Methods

1. In vitro kinase assay

Recombinant human Prak was expressed in *E. coli* as His6-tagged protein substantially as described for Mnk1 by Tschopp et al. (Tschopp et al. (2000) *Molecular Cell Biology Res. Comm.* 3, 205-211). Phospho-p38a and His6-Hsp27 were prepared substantially as described (New et al. and Tschopp et al. *ibid*). All kinase reactions were performed with the following reaction buffer. Kinase buffer (5x): 125 mM Hepes (Stock at 1M; Gibco #15630-056), 125mM β -glycerophosphate (Sigma #G-6251):125 mM MgCl₂ (Merck #5833); 0.5 mM Sodium orthovanadate (Sigma #5-6508), 10 mM DTT (Boehringer Mannheim #708992), pH 7.4. The (5x) kinase buffer is freshly prepared from 25 x stock solution. DTT is stored frozen at -20°C in small aliquots and added immediately before use.

Prior to the assay, recombinant Prak is phosphorylated by incubation with

phospho-p38. The kinase mix is prepared by mixing phospho-p38 (final concentration: 30 μ g/ml) and Prak (final concentration: 150 μ g/ml) in 1 x kinase buffer (containing 5 μ M ATP. The reaction mixture is incubated for 30 min at RT and chilled on ice until further use.

For the assay, the activated kinases are prediluted in 1 x kinase buffer/0.00025% TWEEN20 to 15 μ g/ml.

The compounds to be tested are prediluted in 10% DMSO to 10x the final assay concentration.

The substrate mix is prepared by mixing His-HSP27 (71.5 μ g/ml) with ATP (1.43 μ M) and γ^{33} P-ATP (7.15 μ Ci/ml) in 1 x kinase buffer/0.00025% TWEEN20. To this mix, 5 μ l of compound are added, and the reaction is started by addition of 10 μ l of kinase mix.

The assay is stopped after one hour of incubation at room temperature by the addition of 5 μ l EDTA 0.5M. The label incorporated into protein is separated from labelled ATP by filtration through an Immobilon-P membrane. After washing and drying the membrane, incorporated label is quantified by liquid scintillation counting. Percent inhibition is calculated relative to control reactions which do not contain inhibitor.

2. TNF α and IL-1 release from human PBMCs

Cell separation and culture

All cell culture and ELISA work was done on 96 well plates (Costar). Mononuclear cells were prepared from the blood of healthy volunteers using ficoll-hypaque density separation according to the method of Hansel et al (Hansel TT et al. (1991) An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. J. Imm. Methods 145, 105-110) and used at a concentration of 100,000 cells/well in RPMI 1640, 5 % FCS (Gibco). Serial dilution of the test compound were made in DMSO and diluted with culture medium. Final DMSO concentration in the culture medium was 0.1 %. Compound were preincubated with the cells for 30 minutes at 37 $^{\circ}$ C, 5 % CO₂. Then, the cells were stimulated with LPS (5 μ g/ml, Sigma) and γ -interferon (100 U/ml, Boehringer Mannheim) and cultured for 3 hours (for TNF α -determination) or 4.5 hours (for IL-1 β and IL-6 determination). The plates were centrifuged, the supernatants were removed and stored at -80 $^{\circ}$ C until ELISA determination.

TNF α - ELISA

For ELISA determination, supernatants were diluted 1:1 and added into a plate coated with the anti humanTNF monoclonal antibody (357-101-4, clone 92030603 from European Collection of Animal Cell Cultures, antibody produced and purified within Novartis Pharma, 1 μ g/ml in PBS) After overnight incubation, the plates were washed (4x) and biotinylated anti humanTNF monoclonal antibody 2-179-E11 (clone 92030602 (European Collection of Animal Cell Cultures), produced and purified in Novartis Pharma) was added to a final concentration of 1 μ g/ml (100 μ l/well). After 4 hours of incubation, plates were washed 4 x and streptavidin-alkaline phosphatase (Jackson ImmunoResearch, #016-050-084, 1:7500 dilution) was added. The p-nitrophenyl phosphate substrate was prepared immediately before the assay from tablets (1 mg/ml in buffer pH 9.8, 100 μ l/well). Color formation was monitored in a Bio-Tec ELx 808 plate reader at 405 nm in the kinetic mode for 30 minutes. A set of humanTNF standards (31.1 pg - 2000 pg) is included on each plate and used to calculate TNF concentrations from the slopes for each individual well. IC₅₀ -values were calculated using the Origin software package by weighted fitting of the means of percent inhibition for each inhibitor concentration to the logistic function.

Hu IL-1 β ELISA

ELISA microtiter plates were coated with a murine anti-human IL-1 β MAb (Human IL-1 β Cytoset (Biosource International Inc., #CHC1214), 100 μ l at 1 μ g/ml) in PBS 0.02% NaN₃ and incubated overnight at +4 °C. The following day, microtiter plates were washed 4 times with PBS/ 0.05% Tween/ 0.02% NaN₃ and blocked with 300 μ l of PBS/ 2% bovine serum albumin (BSA)/ 0.02% NaN₃ for 3 h. Plates were washed again (4 times) and 100 μ l of supernatant (final dilutions of 1:15 and 1:30) or of the recombinant human IL-1 β standard (titration curve ranging from 640 to 10 pg/ml in 2 fold dilution steps) was added in duplicate together with a biotin-labelled murine anti-human IL-1 β MAb (50 μ l at 0.4 μ g/ml). The plates were incubated for 2 h at room temperature with shaking (700 rpm). After incubation the plates were washed 4 times, and streptavidin alkaline phosphatase conjugate (Jackson ImmunoResearch, #016-050-084) was added at a final dilution of 1/3000 (100 μ l/well; 30 min at room temperature). After washing (4 times) the substrate (p-nitrophenylphosphate in diethanolamine buffer; 100 μ l) was added for 30 min. Reaction was blocked by the addition of 50 μ l/well of NaOH. Plates were read in a microtiter reader (Bio-Rad) using filters of 405 and 490 nm.

3. Cytotoxicity

THP-1 cells are obtained from ATCC and stored in liquid nitrogen. Medium for growth of THP-1 cells: RPMI 1640(GIBCO) + 10 % fetal bovine serum (GIBCO) + L-glutamine (2 mM) + penicillin/streptomycin (100 U/ml) + β -mercaptoethanol (0.05 mM). Cell culture medium for THP-1 incubation: RPMI 1640(GIBCO) + 5 % fetal bovine serum (GIBCO) + L-glutamine (2 mM) + penicillin/streptomycin (100 U/ml) + β -mercaptoethanol (0.05 mM).

The solution for stimulation is prepared by mixing equal volumes of IFN γ (Boehringer Mannheim, 2000 U/ml) and LPS (Sigma; L8274, 100 μ g/ml). The dye solution is prepared from 500 mg MTT (Sigma, M2128) in 100 ml phosphate buffered saline (PBS) and kept under sterile conditions at 4 °C. Sodium dodecyl sulfate (SDS) solution (10 %) is made from 50 g SDS (Bioprobe Systems) and 5 ml 1N HCl. The solution is made up to 500 ml with distilled water and kept at room temperature (precipitates in the fridge). All incubations are done at 37°C in 5 % CO₂.

Test compounds are dissolved in dimethylsulfoxide (DMSO) at 10 mM and further diluted in DMSO to 3 mM and 1 mM. The DMSO stock solutions are further diluted in RPMI 1640 to 300 μ M, 100 μ M and 30 μ M. The final DMSO concentration is 0.1 or 0.3 %.

The 96-well plates for the incubation are prepared as follows: In duplicate wells, 60 μ l cell culture medium, 20 μ l compound and 100 μ l THP-1 cell culture (500.000 cells/ml) are pre-incubated for 30 min.

To stimulate the cells, 20 μ l of the LPS/ γ -IFN solution is added and the cells are incubated for 24 hours.

At the end of the incubation period, the plates are centrifuged and 100 μ l medium is removed. Dye solution (10 μ l) is added to each well and the plates are incubated for 4 hours or overnight. SDS-solution (100 μ l) is added to each well and the plates are again incubated for at least 10 hours.

The absorbance at 540 nm is read with a microplate reader (Biotec 808 Elx). Blue-colored wells indicate living cells, whereas yellow wells indicate a high percentage of dead cells.

Calculation of cytotoxicity: The absorbance corresponding to 100 % cytotoxicity equals the absorbance of wells without cells (no MTT converted into the blue dye). The absorbance corresponding to no cytotoxicity is taken from wells without substance, but with control solvent. Percent cytotoxicity is calculated for each substance concentration.

4. IL-6 production in human dermal fibroblasts

Neutralization assay

Human dermal foreskin fibroblasts were obtained from Clonetics (CC-2509) and grown in FBM (Clonetics, CC-3131) including bFGF (1 ng/ml, CC-4065), insulin (5 μ g/ml, CC-4021), and 2% FCS (CC-4101).

For induction of IL-6, cells were seeded at a density of 10^4 cells per well in a 48 well tissue cluster. The following day, cells were starved for 6-7 h in FBM containing 2% FCS. Compounds were added at final concentrations of 10 and 1 μ M 30 min prior to stimulation with recombinant human IL-1 β (100pg/ml).

Cell supernatant was taken 16-17 h after stimulation and the amount of released

IL-6 determined in a sandwich ELISA.

IL-6 ELISA

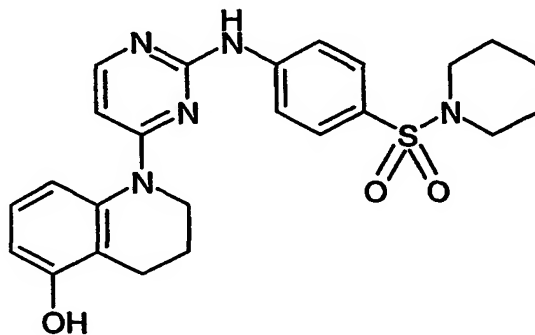
ELISA microtiter plates were coated with a murine anti-human IL-6 MAb (314-14 (Novartis Pharma; batch EN23,961, 5.5 mg/ml); 100 μ l at 3 μ g/ml) in PBS 0.02% NaN₃ and incubated overnight at +4 °C. The following day, microtiter plates were washed 4 times with PBS/ 0.05% Tween/ 0.02% NaN₃ and blocked with 300 μ l of PBS/ 3% bovine serum albumin (BSA)/ 0.02% NaN₃ for 3 h. Plates were washed again (4 times) and 100 μ l of supernatant (final dilutions of 1:20) or of the recombinant human IL-6 standard ((Novartis Pharma #91902), titration curve ranging from 1 to 0.0156 ng/ml in 2 fold dilution steps) was added in duplicate. After an overnight incubation at RT the plates were washed (4 times) and a different murine anti-human IL-6 MAb (110-14, Novartis Pharma; 6.3 mg/ml); 100 μ l at 1 μ g/ml; 3 h at room temperature) was added. After additional 4 washes, a biotin-labelled goat anti-mouse IgG2b antiserum (Southern Biotechnology; #1090-08) was added at the final dilution of 1/10000 (100 μ l/well; 3 h at room temperature). After incubation plates were washed 4 times and streptavidin coupled to alkaline phosphatase (Jackson Immunoresearch, #016-050-084) was added at a final dilution of 1/3000 (100 μ l/well; 30 min at room temperature). After washing (4 times) the substrate (p-nitrophenylphosphate in diethanolamine buffer; 100 μ l) was added for 30 min. Reaction was blocked by the addition of 50 μ l/well of 1.5 M NaOH. Plates were read in a microtiter reader (Bio-Rad) using filters of 405 and 490 nm. IL-6 levels in culture supernatants were calculated in reference to the standard curve using the cubic curve fit. Percentage inhibitions and IC₅₀ values were calculated

Results

As shown in the Table, a small molecular weight compound which inhibits the kinase activity of Prak in vitro, inhibit with a similar IC₅₀ the release of TNF α and IL-1 β from LPS-treated human peripheral mononuclear cells. In addition, the production of IL-6 is inhibited in human dermal fibroblasts by this compound.

Compound	In vitro kinase assay Prak IC ₅₀ [nM]	TNF α release IC ₅₀ [nM]	IL-1 β release IC ₅₀ [nM]	Cytotoxicity IC ₅₀ [nM]	Inhibition of IL-6 @ 1 μ M [%]
Compound A	1100	2200	1100	>30000	100

Compound A has the following structural formula

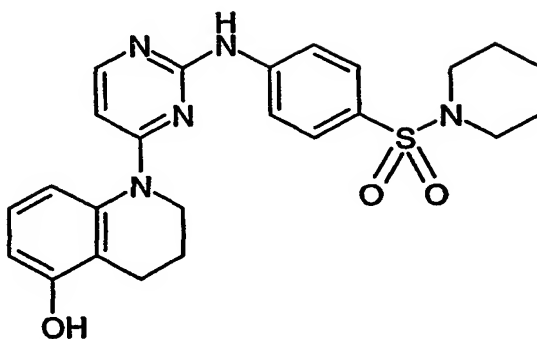


Compound A is included per se within the scope of the present invention.

CLAIMS

1. Use of PRAK for the identification of a TNF Modulator.
2. Method for the identification of a TNF Modulator which comprises contacting a test compound with a system comprising a PRAK protein and monitoring the system for inhibition of PRAK catalytic activity.
3. Method for the comparison of TNF Modulators, comprising separately contacting the TNF Modulators with a system comprising a Prak and comparing each system for inhibition of PRAK catalytic activity.
4. A TNF Modulator when identified by a method according to claim 2.
5. A TNF Modulator when selected by a method comprising the method of claim 3.
6. The use of a TNF Modulator according to claim 4 or 5 for the prophylaxis and treatment of diseases or pathological conditions mediated by cytokines such as TNF α , IL-1 or IL-6.
7.
 - i) A method of reducing inflammation in a subject in need of such treatment which method comprises administering to said subject an effective amount of a TNF Modulator according to claim 4 or 5;
 - ii) A TNF Modulator according to claim 4 or 5 for use as a pharmaceutical;
 - iii) A pharmaceutical composition comprising a TNF Modulator according to claim 4 or 5 in association with a pharmaceutically acceptable diluent or carrier, or
 - iv) Use of a TNF Modulator according to claim 4 or 5 in the manufacture of a medicament for use as an immunosuppressant or anti-inflammatory.
8. A TNF Modulator which is a specific inhibitor of PRAK.
9. The use of a TNF Modulator which is a specific inhibitor of PRAK for the prophylaxis and treatment of diseases or pathological conditions mediated by cytokines such as TNF α , IL-1 or IL-6.

10 The compound having the structural formula



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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ASSAY METHOD, TNF MODULATOR AND ITS USE

(57) Abstract: An assay is provided for the identification and comparison of compounds having activity as modulators of the expression and release of TNF α , IL-1 or IL-6 in humans and animals in which modulation of the catalytic activity of PRAK (p38-regulated/activated protein kinase) is employed for identification and comparison of test compounds.



WO 02/056888 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/00406

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K31/505 A61P29/00 G01N33/68 C12Q1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, MEDLINE, WPI Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NEW LIGUO ET AL: "PRAK, a novel protein kinase regulated by the p38 MAP kinase." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 17, no. 12, 15 June 1998 (1998-06-15), pages 3372-3384, XP002213775 ISSN: 0261-4189 the whole document --- -/--	1-10

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

23 September 2002

Date of mailing of the international search report

10/10/2002

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/00406

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NI HONG ET AL: "MAPKAPK5, a novel mitogen-activated protein kinase (MAPK)-activated protein kinase, is a substrate of the extracellular-regulated kinase (ERK) and p38 kinase." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 243, no. 2, 13 February 1998 (1998-02-13), pages 492-496, XP002213776 ISSN: 0006-291X the whole document	1-10
A	LARSEN JANICE K ET AL: "Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle." AMERICAN JOURNAL OF PHYSIOLOGY, vol. 273, no. 5 PART 1, November 1997 (1997-11), pages L930-L940, XP002213777 ISSN: 0002-9513 the whole document	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/00406

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 6, 7 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-9 relate to compounds defined by reference to a desirable characteristic or property, namely " a TNF modulator". The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only one of such compounds (compound of claim 10). In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a function. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Furthermore, it is impossible to derive from the prior art which compound has such a function because said prior art does not necessarily disclose it. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the kinase assay disclosed in the application as well as for the compound of claim 10.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

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